

A Direct Interaction of Axonin-1 with NgCAM-related Cell Adhesion Molecule (NrCAM) Results in Guidance, but not Growth of Commissural Axons

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Abstract. An interaction of growth cone axonin-1 with the floor-plate NgCAM-related cell adhesion molecule (NrCAM) was shown to play a crucial role in commissural axon guidance across the midline of the spinal cord. We now provide evidence that axonin-1 mediates a guidance signal without promoting axon elongation. In an in vitro assay, commissural axons grew preferentially on stripes coated with a mixture of NrCAM and NgCAM. This preference was abolished in the presence of anti-axonin-1 antibodies without a decrease in neurite length. Consistent with these findings, commissural

axons in vivo only fail to extend along the longitudinal axis when both NrCAM and NgCAM interactions, but not when axonin-1 and NrCAM or axonin-1 and NgCAM interactions, are perturbed. Thus, we conclude that axonin-1 is involved in guidance of commissural axons without promoting their growth.

Key words: axon guidance • growth cone • neuronal cell adhesion molecules • immunoglobulin superfamily • signal transduction

Introduction

Growing axons find their target area by integrating positive and negative guidance cues along their trajectory. The molecules serving as guidance cues are secreted from intermediate or final targets, or are displayed on the surface of cells or in the extracellular matrix along the axons' pathway. The guidance function of these molecules consists of a diverse set of effects, ranging from promotion of axon elongation to induction of growth cone collapse followed by axonal retraction (Tessier-Lavigne and Goodman, 1996). The complex pattern of axonal highways with intersections and bifurcations is encoded by a wealth of molecular cues mediating axon growth, fasciculation, and guidance. Therefore, axon pathfinding can be seen as the result of axon growth along these highways combined with specific decisions at choice points (Stoeckli and Landmesser, 1998).

A relatively well characterized choice point is the floor plate, a triangular structure formed by specialized cells at the ventral midline where commissural axons of the spinal cord decide to cross. Commissural axons, located in the

dorsolateral area of the spinal cord, project ventromedially toward the floor plate because they are attracted by netrin-1 (Kennedy et al., 1994; Serafini et al., 1994). To cross the midline, the axons enter the floor plate. After reaching the contralateral border, they turn rostrally, still keeping contact with the floor plate (Bovolenta and Dodd, 1990). Growth across the floor plate is mediated by interactions between cell adhesion molecules (CAMs)¹ of the Ig superfamily that are exposed on the surface of floor-plate cells and the growth cones of commissural axons (for review see Stoeckli and Landmesser, 1998). Axonin-1 is expressed on commissural axons (Shiga and Oppenheim, 1991; Stoeckli and Landmesser, 1995), whereas NgCAM-related cell adhesion molecule (NrCAM) is displayed on floor-plate cells (Krushel et al., 1993; Moscoso and Sanes, 1995; Stoeckli and Landmesser, 1995). When antibodies against axonin-1 or NrCAM were injected into the central canal of the embryonic chicken spinal cord in vivo, commissural axons committed pathfinding errors (Stoeckli and Landmesser, 1995). Instead of growing across the floor

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¹Abbreviations used in this paper: CAM, cell adhesion molecule; Fab, antigen binding fragment; FnIII, fibronectin type III; NgCAM, neuron-glia cell adhesion molecule; NrCAM, NgCAM-related cell adhesion molecule.

plate to join the contralateral longitudinal tract, a considerable number of axons prematurely joined the ipsilateral longitudinal tract. Because injections of anti-axonin-1 and anti-NrCAM produced a similar pattern of pathfinding errors *in vivo*, and because purified axonin-1 and NrCAM bound each other *in vitro* (Suter et al., 1995), it was suggested that a direct binding of the growth cone axonin-1 with floor-plate NrCAM was required for guiding commissural axons across the floor plate. In the absence of axonin-1 and NrCAM interactions, the ipsilateral longitudinal tract obviously presented an alternative that had been completely neglected in the unperturbed choice situation.

In the present study, we have focused in more detail on the molecular and functional interaction of axonin-1 and NrCAM and its impact on the choice of commissural axons to grow across the floor plate. Based on the ability of axonin-1 to bind both NgCAM and NrCAM *in vitro* (Kuhn et al., 1991; Suter et al., 1995), and based on the strong expression of NrCAM on floor-plate cells and NgCAM on axons of the ventral longitudinal tract (Shiga et al., 1990; Shiga and Oppenheim, 1991), we speculated that commissural axons at the floor-plate border choose between two pathways of growth-promoting molecules: an NrCAM pathway across the floor plate and an NgCAM pathway along the ipsilateral longitudinal tract. Normally, commissural axons opt for the NrCAM pathway, and only under experimental conditions, when the NrCAM pathway is not accessible, the NgCAM pathway is chosen. Therefore, we postulated that commissural growth cones grow across the floor plate, because they prefer NrCAM over NgCAM as a growth-promoting substratum. To test this hypothesis, we used a stripe assay, in which the axons emerging from explants of commissural neurons were offered the choice to elongate either on NrCAM or on NgCAM. To our surprise, the commissural axons showed no preference, but grew equally well on NgCAM and NrCAM substratum. However, a clear preference was observed for a mixed NrCAM/NgCAM substratum that was alternated with an NgCAM substratum. In the presence of anti-axonin-1 antibodies, this preference was abolished. Because the same anti-axonin-1 antibodies that blocked the preference of commissural axons for the mixed substratum did not reduce neurite lengths on NrCAM, NgCAM, or mixed NrCAM/NgCAM substratum, we concluded that axonin-1 is important for axon guidance but not for axon growth. Thus, guidance of commissural axons across the floor plate is not achieved by selecting the most potent growth-promoting substrate, but rather guidance and growth are distinct functions mediated by Ig superfamily CAMs. In accordance with exhibiting distinct functions, CAM-CAM interactions can differ in their topology. Whereas the cis-interaction between axonin-1 and NgCAM appears to be involved in fasciculation of commissural axons, a trans-interaction between axonin-1 and NrCAM is essential for their guidance across the midline.

Materials and Methods

Choice Assay with Commissural Neuron Explants

Commissural neurons were dissected from the lumbosacral spinal cord of chicken embryos at stage 19–20 (Hamburger and Hamilton, 1951) as de-

scribed previously by Stoeckli et al. (1997). The neurons were dissected before they had established contact with the floor plate *in vivo*, and, thus, the axons grown in culture corresponded to the first axons developed by the commissural neurons, rather than to the regenerating axons. The stage of the embryos used was critical since commissural neuron explants prepared from embryos at stage 21 or later were no longer able to attach to NrCAM, NgCAM, or mixed NrCAM/NgCAM substrata.

NrCAM and NgCAM were immunoaffinity-purified from brain membranes of 14-d-old embryos using the mAbs 2B3-C8 against NrCAM (de la Rosa et al., 1990; Kayyem et al., 1992a) and 12-I-14-E 311 against NgCAM (Rathjen et al., 1987). The purity of NrCAM and NgCAM was tested by SDS-PAGE and Western blotting. We found no traces of NgCAM and axonin-1 in purified NrCAM, and purified NgCAM was free of contamination by NrCAM or axonin-1 (data not shown).

Commissural neurons were grown in a netrin-1-containing serum-free medium. Conditioned medium was obtained by growing netrin-1-secreting 293-EBNA cells (provided by Drs. Christine Mirzayan and Marc Tessier-Lavigne, Howard Hughes Medical Institute, University of California San Francisco, San Francisco, CA) for 3 d in DME/F12 (Sigma Chemical Co.) supplemented with 5 mg/ml Albumax (GIBCO BRL), 100 μ g/ml transferrin, 10 μ g/ml insulin, 20 ng/ml triiodothyronine, 40 nM progesterone, 200 ng/ml corticosterone, 200 μ M putrescine, 60 nM sodium selenite, 1 mM sodium pyruvate, and 2 mM glutamine (all from Sigma Chemical Co.). The conditioned medium was harvested, passed through a 0.22- μ m filter, and diluted one to one with freshly prepared DME/F12 with all the supplements described above. For antibody perturbation experiments, the medium was supplemented with 250 μ g/ml rabbit anti-NrCAM Fab of polyclonal serum R51, 250 μ g/ml goat anti-NgCAM Fab of polyclonal serum G4 (Kuhn et al., 1991), or 500 μ g/ml goat anti-axonin-1 IgG of polyclonal serum G67. IgG and Fab were prepared as previously specified (Stoeckli et al., 1991). In the presence of anti-NrCAM antibodies, only very few explants successfully attached to the NrCAM substratum and neurite growth was strongly inhibited (not shown). The same result was obtained with anti-NgCAM antibodies on an NgCAM substratum. To exclude cytotoxicity of the polyclonal antibodies used, we added anti-NrCAM antibodies to cultures on NgCAM and vice versa. Under both conditions, explants attached well, and no reduction of neurite length was observed (not shown).

For the choice assay, stripes of different substrata were coated directly onto tissue culture plastic (Nunc, Inc.) using a special silicone matrix (provided by Ulrike Binkle and Dr. Claudia A.O. Stuermer, Department of Biology, University of Konstanz, Konstanz, Germany). The stripes were prepared according to the procedure described by Vielmetter et al. (1990). In brief, for the first stripes, 10 μ g/ml immunoaffinity-purified NgCAM or NrCAM was injected into the open channels of the matrix that was placed onto the tissue culture plastic. After incubation for 1 h at 37°C, the injections were repeated. After another hour of incubation at 37°C, unbound proteins were removed by rinsing the dishes twice with PBS. For alternating stripes of two different substrata, the remaining binding sites were saturated with a blocking solution containing 5 mg/ml Albumax in PBS for 30 min at 37°C. This blocking step was omitted, when the second substratum was coated uniformly. The blocking solution was exchanged twice for PBS and the matrix was removed before the dish was incubated with the second substratum (immunoaffinity-purified NrCAM or NgCAM, each 10 μ g/ml) for 2 h at 37°C. When alternating stripes of NrCAM and NgCAM were coated, the order of coating could be changed without an effect on the result. To minimize the volume of the medium, the area of the culture dish used for the explants was limited by a grease ring (high vacuum grease; Dow Corning) that was applied by using a 14-ml Falcon snap-cap tube as a stamp. Commissural neuron explants were cultured for 40 h at 37°C before fixation in 2% formaldehyde and 0.05% glutaraldehyde. The fixative was added directly to the culture medium as a concentrated solution. Fixation was at 37°C for 30 min. In all cultures, the coated stripes were visualized by indirect immunofluorescence. The following antibodies were used: for NrCAM, the polyclonal rabbit serum R51 or the mAb 2B3-C8; and for NgCAM, the polyclonal rabbit serum R24 (Kuhn et al., 1991) or the mAb 12-I-14-E 311. As secondary antibodies, we used FITC-conjugated goat anti-rabbit IgG (Cappel Laboratories; Organon Teknica) and Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.).

Neurite Outgrowth Assay

For neurite outgrowth assays, explants of commissural neurons were grown on 8-well LabTek Permanox slides (Nunc) coated with immunoaf-

finity-purified NrCAM, NgCAM, or mixed NrCAM/NgCAM (each 10 μ g/ml) for 2 h at 37°C and washed twice with PBS. Commissural neuron explants were cultured for 18 h at 37°C and fixed in 2% formaldehyde and 0.05% glutaraldehyde as described above. For quantitative analysis, the longest (3–5) neurites of each explant were measured using an inverted microscope equipped with phase-contrast optics and the image analysis software NIH Image. For each condition, cultures of three independent experiments were analyzed. Because the neurite lengths measured were not significantly different (Kruskal-Wallis test; Stat View, version 4.51), the results were pooled. The percentage of neurites longer than a given length was plotted versus neurite length (Chang et al., 1987).

Quantification of Growth Cone Areas of Commissural Axons

For quantification of the growth cone areas, single growth cones were identified and measured using the NIH Image analysis software (version 1.61). At least three independent experiments were analyzed for each condition. Because the values were not significantly different (Wilcoxon rank sum test), the results of the experiments were pooled. The growth cone areas measured under different conditions were compared in the Splus statistics program.

In Vivo Perturbation of Axonin-1, NrCAM, and NgCAM Interactions

In vivo injections and analyses of commissural axon pathfinding were done as described previously (Stoeckli and Landmesser, 1995). Small volumes of solutions containing one or combinations of two or three different antibodies (10 mg/ml each) were injected into the central canal of the developing spinal cord in ovo. Injections were repeated every 8 h between stages 18 and 23, which is the time when commissural axons in the lumbosacral region project to the floor plate and cross the midline. The embryos were killed between stage 25 and early 26. Commissural axon growth and pathfinding behavior were analyzed in whole-mount preparations (open books) by injections of FastDiI (1,1'-dilinoylel-3,3,3',3'-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate; Molecular Probes, Inc.) into the area of neuronal cell bodies.

Heterologous Expression of NrCAM in Myeloma Cells

For aggregation assays, NrCAM was expressed in the nonadherent cell line J558L according to the protocol described earlier for the expression of axonin-1 (Rader et al., 1993). The full-length cDNA of NrCAM was constructed from three different PCR fragments using the following primers: AB6F, 5'-AGTAGAGCTCGCCACCATGATGAAAGAGAAGAG-3' (SacI underlined, start codon boldfaced); AB21B, 5'-CTTCTCCTGGTACCATGAGAGTTC-3'; Nr-1F, 5'-TTCTCGAAGCTTCTTTGTGTAAAGGG-3' (HindIII underlined); and Nr-1B, 5'-AGGGATCCCTTACACAAATGATTCATGGCATTAC-3' (BamHI underlined, termination codon boldfaced). The PCR fragments were ligated, using 175 bp from the AB6F-AB21B fragment (cut at the primer-derived SacI and an internal XhoI site at position 196) and the 3,148-bp fragment (Robert P. Lane; from XhoI [196] to HindIII [3,278] including AS12 described by Kayyem et al. [1992b]). The 562-bp Nr1F-Nr1B fragment was attached using the HindIII site (3,278) and the BamHI site of the backward primer. The full-length cDNA was cloned into the vector pCD4-FvCD3-c_κ (Traunecker et al., 1991), which was modified by introducing a multiple cloning site and the splice donor consensus sequence GTAAGT between the SacI and the HindIII sites. Stable transfectants of mouse myeloma cells J558L were obtained by protoplast fusion and selection with L-histidinol (Rader et al., 1993). After 10 d, the cells were screened for surface NrCAM by indirect immunofluorescence. Clones with high NrCAM expression were expanded and subcloned.

Cell Aggregation Assay

CAM-expressing myeloma cells were labeled with different intracellular fluorescent dyes to follow their aggregation pattern. For the aggregation assay, we used the procedure described by Rader et al. (1993) with the following modifications: after 30 min at 37°C, the cells were fixed in a final concentration of 2% formaldehyde and 0.05% glutaraldehyde for 30 min at room temperature. To reduce the background, 1 M ethanolamine (Fluka Chemie) in PBS, pH 7.4, was added to a final concentration of 50 mM. After incubation for 15–20 min at room temperature, freshly pre-

pared 1 M sodium borohydride (Sigma Chemical Co.) was added to a final concentration of 45 mM, and the cells were incubated for 1 h at room temperature. After centrifugation in v-shaped 96-well plates (Corning Costar Corporation) for 3 min at 1,000 rpm in a Sorvall 6000D, the cells were resuspended in PBS, and examined with a fluorescence microscope (Leitz DMRD; Leica Microsystems) using the G/R filter that allows simultaneous visualization of FITC and TRITC. For controls, the myeloma cells were incubated with either 500 μ g/ml rabbit anti-NrCAM Fab or goat-anti-axonin-1 Fab during loading of the intracellular dyes. For quantitative analysis, the total number of cells (at least 100) within the view field of the microscope, the percentage of cell in aggregates, and the ratio of the two cell types were determined. Results from at least nine independent experiments were statistically analyzed.

Immunoblotting

Stably transfected myeloma cells expressing NrCAM or NgCAM were washed twice with PBS and counted. Transiently transfected COS7 cells were analyzed 2 d after electroporation. The cells were washed twice with PBS, detached and dissociated by treatment with 2 mM EDTA in PBS, and were counted. To solubilize the membrane proteins, the cells were lysed in 1% (wt/vol) Triton X-100, 1% (wt/vol) CHAPS, 0.1% (wt/vol) SDS, 5 mM EDTA, 1 mM PMSF, 1 mM iodoacetamide, 100 mM NaCl, and in 50 mM Tris-HCl, pH 7.5. The solubilized proteins were precipitated according to Wessel and Fluegge (1984) and resuspended in sample buffer. After boiling for 5 min, the proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The nitrocellulose was blocked overnight in 1% (wt/vol) blocking reagent for chemiluminescence detection (Roche Diagnostics) in TBS and incubated for 2 h in 1% (wt/vol) blocking reagent/TBS containing the primary antibody. For detection of NrCAM and NgCAM in myeloma cells, the mAbs 2B3-C8 and 12-I-14-E 311, respectively, were used at a concentration of 10 μ g/ml. For detection of the NrCAM domain deletion mutants expressed in COS7 cells, the rabbit anti-NrCAM antiserum R51 was used at a dilution of 1:2,000. The immunoblots were rinsed thoroughly with TBS before incubation for 1 h in 0.5 μ g/ml sheep anti-mouse Fab or sheep anti-rabbit IgG coupled to peroxidase in 0.5% (wt/vol) blocking reagent in TBS. The membranes were rinsed again in TBS followed by 0.1% (wt/vol) Tween 20 in TBS. Protein bands were detected with the chemiluminescence kit from Roche Diagnostics. For densitometric quantification, the Image QuANT software (version 4.1) from Molecular Dynamics was used.

Construction of NrCAM Domain Deletion Mutants

The domain deletion mutants of NrCAM were inserted downstream of the cytomegaloviral promoter into the eucaryotic expression vector pSCT, which we previously used for the expression of the domain deletion mutants of axonin-1 and NgCAM (Buchstaller et al., 1996; Rader et al., 1996; Kunz et al., 1998). Domain borders were chosen according to Grumet et al. (1991). To construct the domain deletion mutants, three different plasmids were used as a template for PCR: pSCT NrCAM and pBluescript NrCAM, both containing full-length cDNA of chicken NrCAM, and pSP73-Ig1-Fn1 NrCAM (pSP73 from Promega Corporation), which included a truncated form of the NrCAM cDNA coding for all Ig domains and the first FnIII domain. Note that the cDNAs of NrCAM have been cloned in reverse orientation into pBluescript NrCAM and pSP73-Ig1-Fn1 NrCAM plasmids.

The following primer sequences were used for construction of the domain deletion mutants of NrCAM. Bold letters show introduced restriction sites and underlined letters represent mismatches: Ig1F, 5'TGGAGGTTGAGACAATTCTCTAGAGT3'; Ig12B, 5'CCATCTAGATCCCCTTTGTGGACTAAA3'; Kpn1F, 5'TTGTAATGGGACTGTTATTTCTCTCTCTG3'; Ig2dB, 5'AAGCCAGTTACAC**CGG/CCG**CCAGCAGTTCTT3' (EagI restriction site); Ig21F, 5'AGGGGATCTAGAC**CGG/CCG**TATAACAATATT3' (EagI restriction site); Xba1B, 5'GCAGC-TGAAGCTTGCATGCCTGCAGGTGCA3'; Ig23F (dIg34-back), 5'TTC-TGTAAGCTGGCTTGGTTG3'; Ig34B, 5'TACTGGATAACAGCAGCAGC-AGGAAGTTAG3'; Ig34F, 5'TGGGGCAGCTTTTACAGTTACCGA-AAT3'; Ig45B (dIg34-for), 5'CCAAGGATTCTAAGTCTGCG3'; Ig2/3B, 5'AGGCCACCAAGTTCTTCTTACACCAATGGGAGC3'; Ig4/5F, 5'TGGCTCAGCAAGAACATTCACAAATGCATT3'; Ig6F1B, 5'CCA-AATCCACCGCTTGACTTGAATTTGAATTTGACAGGT3'; Ig6-FN1-5', 5'GAACCG**CTCG**ACCAATCCACCGCTTGAC3' (Sall restriction site); BsaBI-3', 5'ATCCCTGTACATTAGAAGG3'; BglII-5', 5'ACAGCCACAGTACAAAGTG3'; Ig6-FN1-3', 5'ACAG**CTCGA**-

CGGTAAATGATAGCTG3', (XhoI restriction site); FN5/TM-5', 5'AGGACTCGAGCAATGGCAAGTCGGCAG3' (XhoI restriction site); and Stop-Sal-3', 5'TAGGGCGAATTGGGTACC3'.

For the construction of Δ Ig1-6 NrCAM, the PCR fragment Ig6-FN1-5'-BsaBI-3' (from pSCT NrCAM as template) was cut with SalI-BsaBI and ligated to the linkers 5'CTAGAAGAATTGTCTCAACC3' (XbaI overhang, sense) and 5'TCGAGGTTGAGACAATTCTT3' (SalI overhang, antisense). The construct was introduced into the expression vector pSCT NrCAM, which had been digested with XbaI-BsaBI. For the construction of Δ Fn1-5 NrCAM, the fragments BglII-5'-Ig6-FN1-3' digested with BglII-XhoI (from pSCT NrCAM) and FN5/TM-5'-Stop-Sal-3' digested with XhoI-SalI (from pBluescript NrCAM) were cloned into pBluescript NrCAM, which had been cut with BglII-SalI. From this vector, an XbaI-SalI fragment was cut and finally cloned into pSCT NrCAM, which was cut with XbaI-SalI. Δ Ig2 NrCAM was generated using a similar strategy. The PCR fragment Kpn1F-Ig2dB (from pSCT NrCAM) was digested with KpnI, treated with polynucleotide kinase, and cloned into pBluescript digested with HincII. An HpaI-EagI fragment was excised from this vector and, together with the PCR fragment Ig21F-Xba1B (from pSP73-Ig1-Fn1 NrCAM; digested with XbaI-EagI), was cloned into pSCT NrCAM, cut with HpaI-XbaI. All other mutants were cloned using the plasmid linearization method, which allows exact domain deletions without amino acid exchanges, because the primers for PCR were matched to the domain borders. As a template for PCR amplifications, we used pSP73-Ig1-Fn1 NrCAM, which was linearized using a restriction enzyme with a unique cutting site within the domain to be deleted. For construction of Δ Ig12, pSP73-Ig1-Fn1 NrCAM was linearized with Bst1107I and the primers Ig1F and Ig2/3B were used for PCR. After ligation and plasmid amplification, an XbaI-BsmI fragment was cloned into pSCT NrCAM digested with XbaI-BsmI. Using the same strategy Δ Ig34 (primer pair, Ig23F and Ig45B), Δ Ig56 (Ig45F and Ig6F1B), Δ Ig1 (Ig1F and Ig12B), Δ Ig3 (Ig23F and Ig 34B), and Δ Ig4 (Ig34F and Ig45B) were generated.

All deletion mutants were verified by double strand DNA sequencing. Based on the numbering of the amino acid sequence of NrCAM used by Grumet et al. (1991), the following segments were deleted in the mutants: Δ Ig1-6 NrCAM, P17-A596; Δ Fn1-5 NrCAM, P598-P1094; Δ Ig12 NrCAM, T18-E217; Δ Ig34 NrCAM, R218-P405; Δ Ig56 NrCAM, P406-R597; Δ Ig1 NrCAM, T18-R111; Δ Ig2 NrCAM, P112-E217; Δ Ig3 NrCAM, R218-P313; and Δ Ig4 NrCAM, Y314-P405. The insertion of the XhoI site into the Δ Fn1-5 NrCAM resulted in a silent mutation of R597 (CGG-CGA). The only mutation generated was R218 (AGG) to P218 (CGG) in Δ Ig12 NrCAM by the insertion of the EagI site.

Expression of NrCAM or Axonin-1 in COS7 Cells

The cDNAs of wild-type and mutant NrCAM or axonin-1 were cloned into the expression vector pSCT and used for transient transfection of COS7 cells according to Rols et al. (1994). In brief, 10^6 COS7 cells were collected by trypsinization and resuspended in 700 μ l PBS. 10 μ g vector DNA purified with the EndoFree plasmid purification kit (QIAGEN GmbH) was added in 100 μ l PBS. The cells were incubated with the DNA for 10 min on ice before electroporation in a 0.4-cm cuvette with a 960- μ F/230 V pulse in a Bio-Rad gene pulser (Bio-Rad Laboratories). After 9.5 min at 37°C, the cells were plated in 60-mm cell culture dishes in DME supplemented with 10% FCS. To remove dead cells and debris, the cultures were washed twice with PBS after 8 h and grown for another 16 h in 10 ml DME supplemented with 10% FCS. This procedure resulted in successful heterologous expression of NrCAM and axonin-1 in 20–30% of the cells. According to the Western blot analysis, all Ig domain deletion mutants were expressed at similar levels (see Fig. 9). The only mutant that was found in lower amounts was Δ Fn1-5 NrCAM. As axonin-1 binding of this mutant was comparable to wild-type NrCAM, the lower expression level did not negatively interfere with the purpose of the study. In summary, the Western blot analysis of the mutant NrCAM proteins revealed the expected molecular masses and demonstrated that the mutant proteins were present on the cells in similar concentrations.

Analysis of Protein-Protein Interactions Using Covaspheres and Transfected COS7 Cells

24 h after transfection, the COS7 cells were washed twice with PBS, harvested by treatment with 2 mM EDTA in PBS, and resuspended in DME with 10% FCS. The cells were transferred to poly-L-lysine-coated glass Lab-Tek slides (Nunc) and cultured for another 24 h. For the Covaspheres-binding assay, the FCS in the culture medium was exchanged for

5 mg/ml Albumax (GIBCO BRL). Axonin-1, NrCAM, and NgCAM were coupled to TRITC- or FITC-labeled fluorescent polystyrene microspheres (Covaspheres, nominal diam 0.5 μ m; Duke Scientific Corp.) as described elsewhere (Kuhn et al., 1991). Immediately before addition to the cells, the Covaspheres were sonicated for 3 min. Transfected COS7 cells were incubated with Covaspheres for 1 h at 37°C. They were rinsed twice with DME-Albumax and fixed with 2% formaldehyde for 1 h at 37°C. After washing twice with 1% FCS in PBS, COS7 cells were subjected to indirect immunofluorescence staining. Wild-type and mutant NrCAM were visualized with the polyclonal goat serum G68 and FITC-conjugated rabbit anti-goat IgG (Zymed Laboratories Inc.). Polyclonal rabbit serum R26 and Texas red-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) were used for detection of wild-type and mutant axonin-1. For examination, we used a Leica microscope (Leitz DMRD). For quantification, strongly fluorescent cells binding at least four Covaspheres were scored as binding cells (nonexpressing cells were found to bind 0–1 Covaspheres).

For antibody perturbation experiments, the cells were preincubated for 1 h with 30 μ g/ml mAbs DIg1, NIg1, and X9H8 raised against axonin-1 (Rader et al., 1996). After rinsing three times with DME-Albumax (5 mg/ml), the Covaspheres were added as described above. For detection of axonin-1-expressing cells, we used a Cy3-conjugated donkey anti-mouse antibody.

Results

Commissural Axons Prefer a Mixed NrCAM/NgCAM Substratum over NgCAM Alone

When commissural axons reach the border of the floor plate *in vivo*, they choose to grow into the floor plate rather than to follow the ipsilateral longitudinal tract. Based on the results of perturbation studies *in vivo* (Stoeckli and Landmesser, 1995) and because NgCAM is highly expressed on axons of the longitudinal tract and NrCAM is expressed on floor-plate cells, we speculated that axonin-1-expressing commissural axons make their pathway choice based on a preference for NrCAM over NgCAM. To test this hypothesis, explants of commissural neurons, characterized by their location within the spinal cord, their responsiveness to netrin-1, and their expression of axonin-1, were cultured on a substratum composed of alternating stripes of NgCAM and NrCAM (for details see Stoeckli and Landmesser, 1995; Stoeckli et al., 1997; see also Materials and Methods). To our surprise, the commissural axons did not show any substratum preference. The axons elongated equally well on both NgCAM and NrCAM, and no differences in axon and growth cone morphologies were detectable (Fig. 1).

However, on close observation of these cultures, we found that the filopodia of many growth cones were touching the border of two adjacent stripes of NrCAM and NgCAM, thus contacting both substrata simultaneously. Therefore, we offered axons a choice between a mixed NrCAM/NgCAM substratum versus NgCAM or NrCAM alone. A clear preference for the mixed NrCAM/NgCAM substratum was seen when commissural axons were given a choice between mixed NrCAM/NgCAM alternated with stripes of NgCAM only (Fig. 2 a). In this situation, the majority of the growth cones was found on the stripes coated with the NrCAM-NgCAM mixture, whereas only occasionally a growth cone was found on an NgCAM stripe. Furthermore, we found that the growth cones residing on the preferred NrCAM/NgCAM stripes were considerably larger than the growth cones growing on alternating stripes of NrCAM and NgCAM (Fig. 2 a, quantified in Fig. 4).

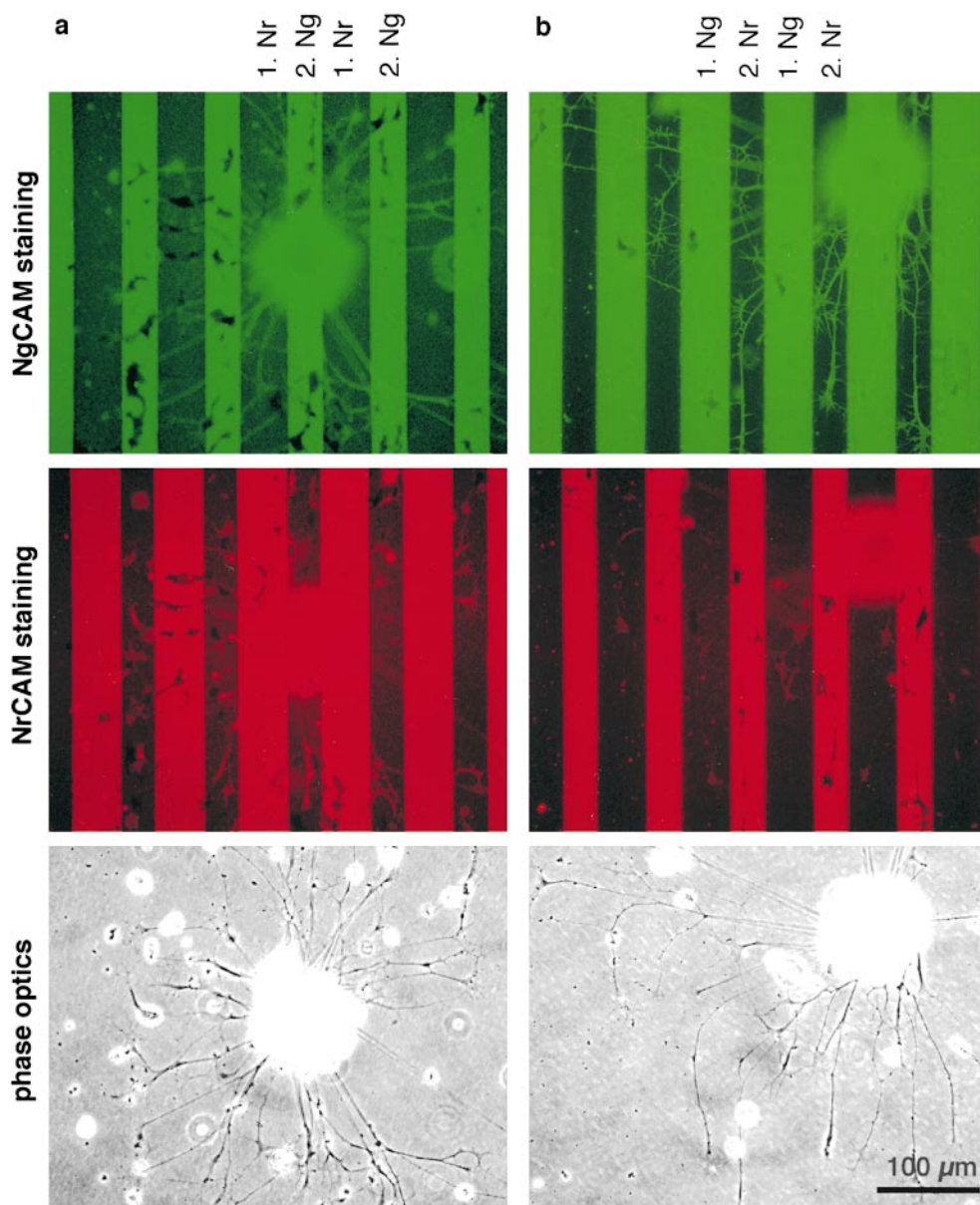


Figure 1. Commissural axons show no preference for plain NgCAM versus plain NrCAM substratum. When commissural explants were cultured on alternating stripes of purified NrCAM and NgCAM, they grew randomly across the stripes. The number of growth cones seen on the red and the green stripes was about equal, indicating that the growth cones did not prefer either one of the substrata. The order of coating could be reversed without an effect on the result (a and b). (a) NrCAM was coated first (1. Nr), followed by NgCAM (2. Ng). (b) The order of coating was reversed: NgCAM was coated first (1. Ng) and NrCAM second (2. Nr). Stripes were visualized with antibodies against NgCAM (first row) and NrCAM (second row). The third row shows the explants with phase-contrast optics.

In contrast, when commissural axons were offered a choice between mixed NrCAM/NgCAM substratum versus pure NrCAM substratum, the growth cones showed no preference and grew randomly without respecting substratum boundaries (Fig. 2 b). The fact that a preference for the mixed NrCAM/NgCAM substratum concomitant with growth cone enlargement was only seen when the alternative substratum was NgCAM, but not when it was NrCAM, excluded a general preference for a composite substratum. Therefore, we concluded that the response of commissural axons in our assay reflected a specific choice.

The Preference of Commissural Axons for the Mixed NrCAM/NgCAM Substratum Depends on Axonin-1

Because previous studies demonstrated a crucial role of axonin-1 and NrCAM interactions in the guidance of commissural axons across the floor plate (Stoeckli and Land-

messer, 1995; Stoeckli et al., 1997), we tested the effects of anti-axonin-1 antibodies in our in vitro choice assay. Interestingly, commissural explants cultured on alternating stripes of NrCAM/NgCAM and NgCAM in the presence of anti-axonin-1 antibodies no longer showed a preference for the mixed substratum (Fig. 3 a). The growth cones were randomly distributed over the entire surface, without respecting substratum borders. They were considerably smaller than those grown on the same substratum combination in the absence of anti-axonin-1 antibodies. For a control, explants were cultured in the presence of anti-NrCAM antibodies (Fig. 3 b). In this case, commissural axons could no longer detect NrCAM in the mixed substratum, and, as expected, grew randomly. As seen in the presence of anti-axonin-1, growth cones were small (Fig. 4). Thus, by either blocking axonin-1 on the growth cones or NrCAM of the substratum, the preference response and the enlargement of the growth cones were abolished.

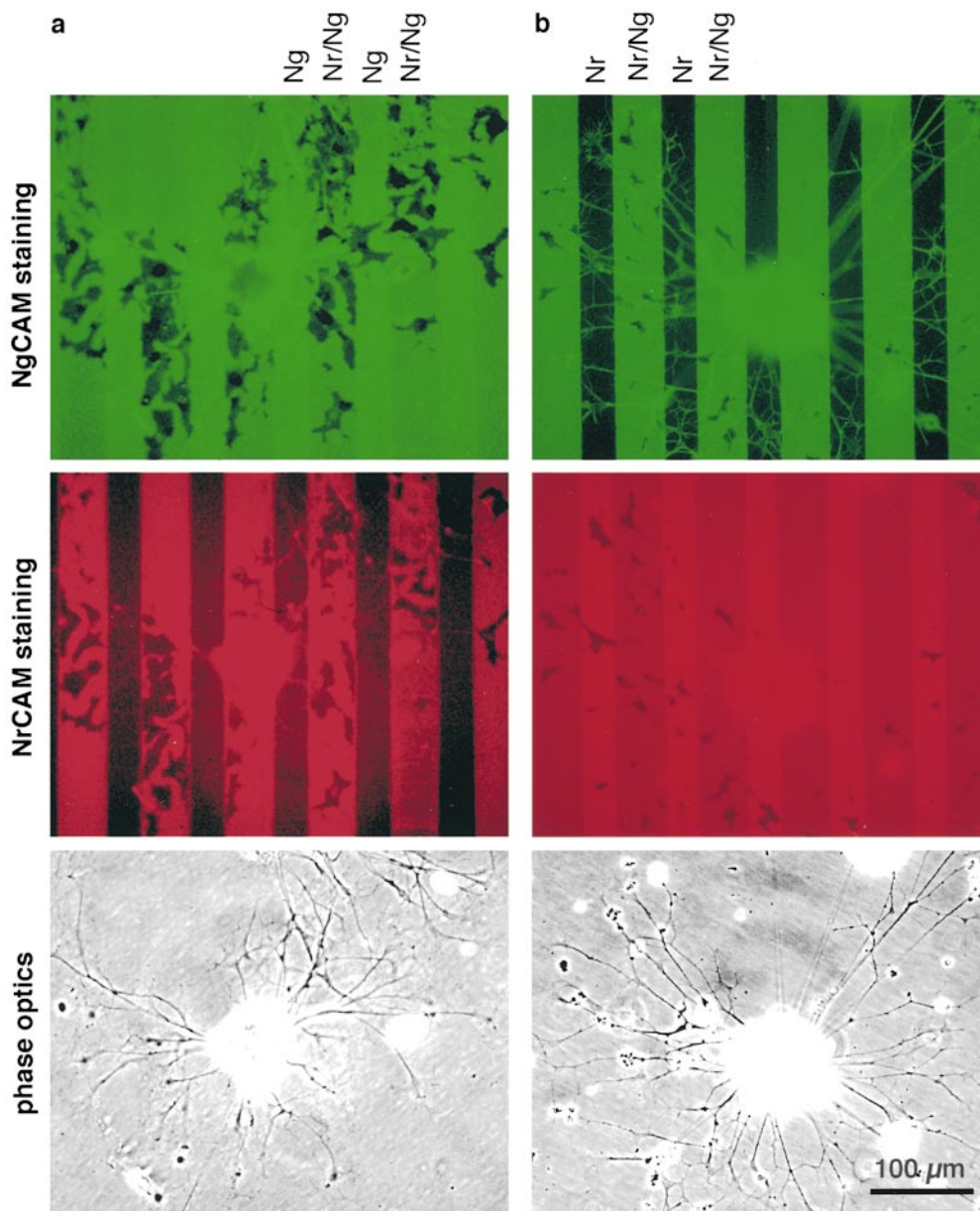


Figure 2. Commissural axons prefer a mixed NrCAM/NgCAM substratum over NgCAM. (a) When commissural axons were offered a choice between stripes with a mixture of NrCAM and NgCAM (Nr/Ng) and stripes with NgCAM (Ng) alone, they clearly preferred the mixed substratum. Growth cones were almost exclusively found on stripes of mixed NrCAM/NgCAM substratum. Note that growth cones were extremely large compared with all other conditions (Figs. 1, 2 b, and 3). Although many growth cones exhibited extended contacts with the border, very few were observed to spread across the border and establish contact with the NgCAM substratum. The pattern of radial outgrowth and the few growth cones observed on NgCAM indicate that growth cones can cross NgCAM stripes. This suggests that the behavior of the growth cones is based on a preference-generating rather than a repulsive effect. Note that large growth cones appear black in the NgCAM and NrCAM staining but can be identified in phase-contrast optics. (b) In contrast, when commissural axons were offered a choice between stripes of mixed NrCAM/NgCAM (Nr/Ng) and stripes of NrCAM (Nr) alone, they showed no preference for the mixed substratum. Growth cones on both types of stripes were smaller than those in a. Substratum stripes were visualized as in Fig. 1.

Enlarged Growth Cones Are only Observed in Conjunction with a Preference Response

Growth cones of the commissural axons on mixed NrCAM/NgCAM substratum alternating with NgCAM were markedly enlarged. Interestingly, growth cones remained small on NrCAM/NgCAM when the alternating stripes were NrCAM or on uniformly coated NrCAM/NgCAM substratum. These observations suggested that the growth cone enlargement and the preference response were connected phenomena. As a first step in addressing this issue, we measured the size of growth cones under eleven dis-

tinct conditions in at least three independent experiments. For each experimental condition, at least 36 single growth cones not contacting an axon or another growth cone were measured with the NIH image analysis program. The results confirmed the impression that only growth cones exhibiting a preference response were significantly enlarged (Fig. 4). Growth cones on mixed NrCAM/NgCAM alternated with NgCAM substratum had an average surface area of 294 μm^2 , whereas, in all other conditions, the average growth cone surface was below 145 μm^2 .

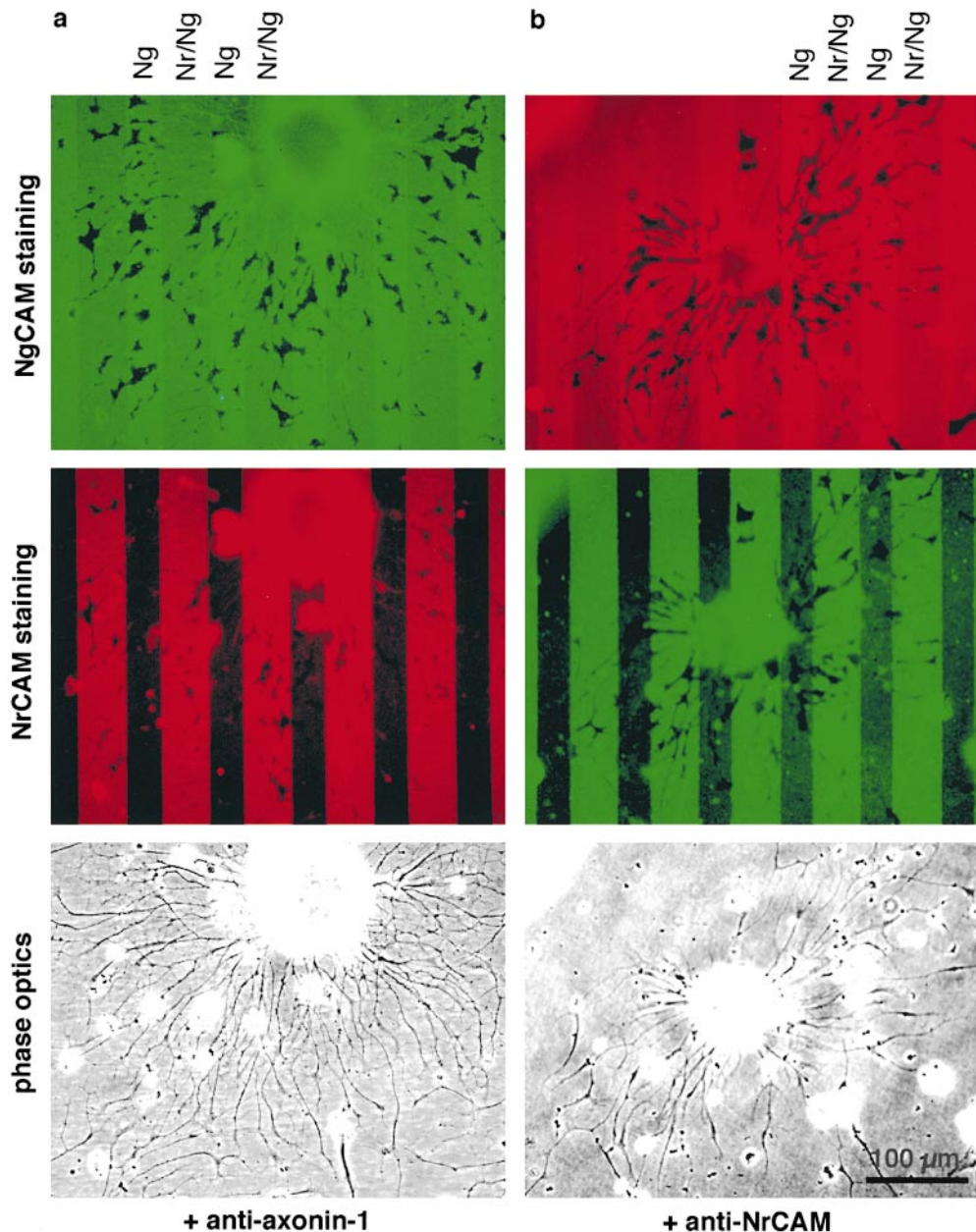


Figure 3. The preference of commissural axons for the mixed NrCAM/NgCAM substratum is dependent on growth cone axonin-1. (a) The presence of anti-axonin-1 antibodies in the culture medium completely abolished the preference of commissural axons for the mixed NrCAM/NgCAM substratum (Nr/Ng) seen in the absence of antibodies (compare Fig. 2 a). Growth cones grew randomly without respecting substratum boundaries. Note that the growth cones are considerably smaller than on the same substratum combination in the absence of antibodies (Fig. 2 a). (b) As a control, anti-NrCAM antibodies were added to the medium of the commissural explants. As expected under these circumstances, NrCAM was no longer detectable as a substratum component, resulting in random neurite growth. Substratum stripes were visualized as in Fig. 1.

Neurite Growth on NrCAM, NgCAM, and Mixed NrCAM/NgCAM Substrata Is Comparable and Not Affected by Anti-axonin-1 Antibodies

To assess the contribution of the axonin-1/NrCAM interaction to neurite outgrowth promotion, we compared neurite lengths on NrCAM, NgCAM, and the mixture of NrCAM and NgCAM in the absence and in the presence of anti-axonin-1 antibodies. As shown in Fig. 5 (a and b), NrCAM, NgCAM, and mixed NrCAM/NgCAM were equally potent in neurite outgrowth promotion. The addition of the same anti-axonin-1 antibodies that were used in the choice assay did not interfere with neurite outgrowth on any substratum (Fig. 5, a and c–e). Therefore, we concluded that the preference-mediating interaction between growth cone axonin-1 and substratum NrCAM is not involved in neurite growth promotion. And conse-

quently, the neurite growth-promoting effect of NrCAM must be mediated by a growth cone receptor distinct from axonin-1.

A Simultaneous Perturbation of NrCAM and NgCAM Interactions Blocks the Extension of Commissural Axons along the Longitudinal Axis In Vivo

We performed a series of in ovo experiments to test the relevance of these in vitro results for commissural axon pathfinding in vivo. A role for axonin-1 and NrCAM in axon guidance was established earlier (Stoeckli and Landmesser, 1995). In these in vivo studies, the perturbation of axonin-1 interactions was shown to result in defasciculation and pathfinding errors of commissural axons. Instead of crossing the midline, axons turned into the longitudinal axis prematurely along the ipsilateral floor-plate border.

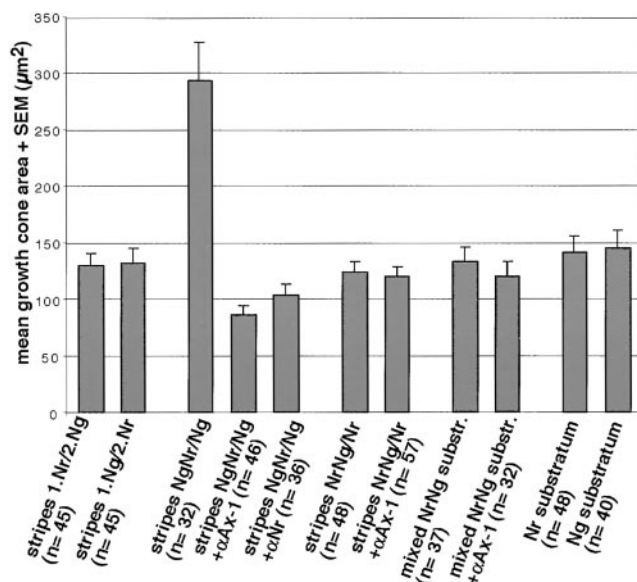


Figure 4. The substratum preference of commissural axons coincides with the enlargement of growth cones. Enlarged growth cones were observed only in the choice situation, where commissural axons clearly preferred mixed NrCAM/NgCAM over single NgCAM substratum (Fig. 2 a). Surface areas of growth cones were measured with the NIH Image analysis 1.61 software. Growth cone areas were $<145 \mu\text{m}^2$ in all conditions, where no preference was observed, such as alternating stripes of NrCAM and NgCAM (first two bars). However, growth cones doubled in size ($x = 294 \pm 34 \mu\text{m}^2$) when a choice for the mixed substratum and against the NgCAM stripes was made. The same substratum combination did not result in enlarged growth cones, when a preference reaction was abolished by addition of either anti-axonin-1 (fourth bar) or anti-NrCAM antibodies (fifth bar). The combination of mixed NrCAM/NgCAM substratum with NrCAM stripes did not elicit a preference correlated with small growth cones (sixth and seventh bars). Accordingly, mixed NrCAM/NgCAM substratum coated homogeneously without or with anti-axonin-1 antibodies did not change growth cone size (eighth and ninth bars). Growth cone areas were comparable on homogeneous NrCAM (10th bar) and NgCAM (11th bar), respectively. The number of growth cones analyzed for each condition is indicated in brackets. Three independent experiments were performed.

Similar pathfinding errors were found after perturbation of NrCAM interactions. In contrast, the perturbation of NgCAM interactions did not result in pathfinding errors. Although commissural axons grew to the ventral border of the spinal cord in a very defasciculated manner, they still managed to cross the midline. Based on these results, a model for commissural axon guidance was suggested in which NgCAM was involved in fasciculation of commissural axons both before and after crossing the midline. NrCAM, expressed by the floor plate, was a binding partner for growth cone axonin-1—mediating entrance and crossing of the floor plate. Thus, axonin-1 was involved in pathfinding and fasciculation of commissural axons, in line with its potential to bind to both NrCAM and NgCAM *in vitro*.

To follow up on the *in vitro* results described above, we performed a new series of *in vivo* experiments, in which we tried to sort out the functional contributions of NrCAM, NgCAM, and axonin-1 to growth and guidance of commissural axons. In particular, we wanted to test whether the contributions of NrCAM and NgCAM were growth promotion, whereas the role of axonin-1 was to select the pathway. To find evidence for the growth-promoting activity of NrCAM and NgCAM, we injected combinations of anti-axonin-1, anti-NrCAM, and anti-NgCAM antibodies into the developing chicken spinal cord *in ovo*. Consistent with the finding that both NrCAM and NgCAM can promote commissural axon growth *in vitro* (Fig. 5), a strong effect on axon growth was found *in vivo* (Fig. 6). The simultaneous blockage of NrCAM and NgCAM interactions resulted in the failure of the majority of commissural axons to extend along the longitudinal axis (Fig. 6, a and e). Many axons made it across the floor plate, and some even turned, whereas others piled up at the ipsilateral border (Fig. 6 e). Similarly, when a cocktail of anti-axonin-1, anti-NrCAM, and anti-NgCAM was injected, axons failed to turn and extend along the longitudinal axis (Fig. 6, b and f). However, due to the additional blockade of axonin-1 interactions, more axons stopped at the ipsilateral border, when all three antibodies were injected (Fig. 6 f). The collapsed, clublike morphology of growth cones treated with the cocktail of all three antibodies is in line with observations made in an earlier *in vitro* study, where growth cones were found to collapse upon floor-plate contact in the presence of anti-axonin-1 but not anti-NrCAM antibodies (Stoeckli et al., 1997). Thus, the *in vivo* results shown here support the hypothesis raised in an earlier study (Stoeckli et al., 1997) that axonin-1 has at least one additional binding partner on the floor plate that is distinct from NrCAM.

When anti-axonin-1 antibodies were injected together with either anti-NgCAM (Fig. 6 c) or anti-NrCAM antibodies (Fig. 6 d), growth along the longitudinal axis was not affected. The most likely explanation for these observations is the redundancy of the growth-promoting activities of NrCAM and NgCAM. When NrCAM is masked, NgCAM takes over and vice versa. As seen *in vitro* (Fig. 5), the growth-promoting activities of NrCAM and NgCAM were equivalent and each molecule stimulated axon growth at the maximal level. The mixture of NrCAM and NgCAM did not have an additive effect. Consistent with results from earlier *in vivo* studies (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997), NrCAM also had an effect on axon guidance. When injected alone or in combination with anti-NgCAM antibodies, anti-NrCAM induced pathfinding errors of some commissural axons. This effect on pathfinding is mediated most likely by a direct interaction with growth cone axonin-1, whereas the growth cone receptor mediating axon growth is not yet identified. Thus, although the situation *in vivo* is more complex than the situation mimicked in our *in vitro* stripe assay, the conclusion drawn from the *in vitro* experiments is valid for the *in vivo* situation. In both cases, axonin-1 is responsible for the choice of the growth cone to grow along one but not the other pathway/stripe without influencing axon extension, because both *in vivo* and *in vitro*, the presence of anti-axonin-1 antibodies did not reduce axon length.

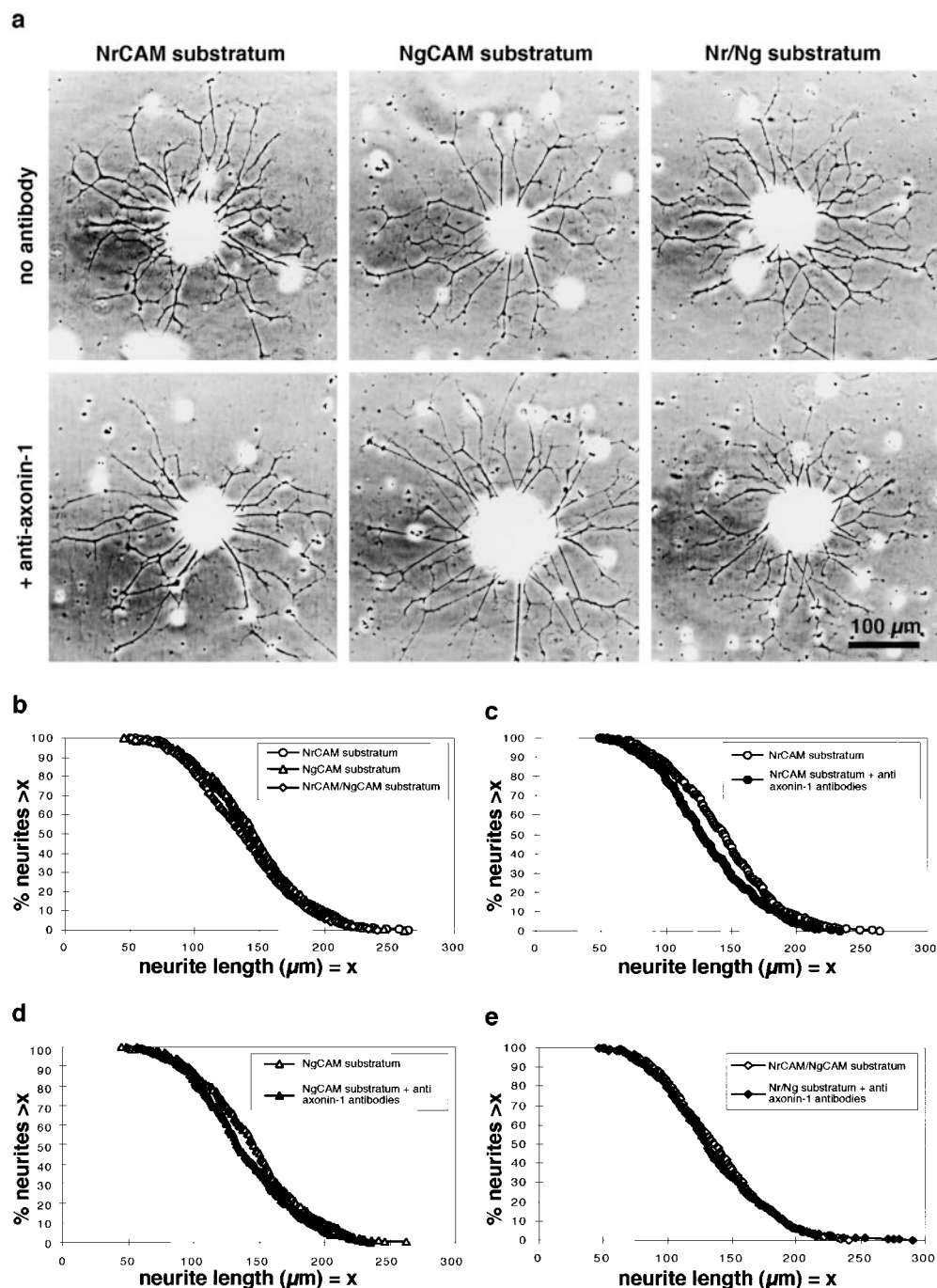


Figure 5. Anti-axonin-1 antibodies do not reduce neurite length on NrCAM, NgCAM, and mixed NrCAM/NgCAM substrata. Commissural neuron explants were cultured on either NrCAM, NgCAM, or mixed NrCAM/NgCAM substrata in the presence or absence of anti-axonin-1 antibodies. After 18 h in culture, the explants were fixed and neurite lengths were analyzed. (a) Representative examples of each experimental condition are shown in phase-contrast optics. No differences in the length and the morphology of the neurites were observed. (b–e) The visual impression was confirmed by quantification of neurite outgrowth. For each experimental condition, at least 20 explants were analyzed. Results of three independent experiments were pooled and plotted as the percentage of neurites longer than a given length versus neurite length (see Materials and Methods for details). (b) The comparison of neurite lengths on NrCAM (open circles), NgCAM (open triangles), and mixed NrCAM/NgCAM (open diamonds) revealed equal neurite outgrowth-promoting activities of all three substrata. The presence of anti-axonin-1 antibodies (filled symbols) in the medium did not reduce neurite length on NrCAM (c), NgCAM (d), or mixed NrCAM/NgCAM (e). Neurite length in the absence of anti-axonin-1 is indicated with open symbols.

The Direct Interaction of Axonin-1 with NrCAM Is a Trans-Interaction

Based on the pathfinding errors of commissural axons caused by injection of antibodies against axonin-1 and NrCAM into the central canal of the spinal cord and based on the expression pattern of axonin-1, expressed by growth cones, and NrCAM, expressed by floor-plate cells, a trans-interaction between the two molecules was suggested (Stoeckli and Landmesser, 1995). A direct interaction between axonin-1 and NrCAM has been described for purified proteins covalently bound to fluorescent polystyrene beads (Covaspheres; Suter et al., 1995), but the to-

poloogy of the axonin-1/NrCAM binding (cis versus trans) has not been investigated so far. A cis- rather than a trans-interaction between axonin-1 and NgCAM had been found to be involved in the growth of dorsal root ganglion neurites (Buchstaller et al., 1996; Kunz et al., 1996; Stoeckli et al., 1996). The effect of both axonin-1 and NgCAM on the fasciculation of commissural axons described in an earlier *in vivo* study (Stoeckli and Landmesser, 1995) is compatible with a cis-interaction between axonin-1 and NgCAM also on commissural axons. However, the role of axonin-1 and NrCAM in commissural axon pathfinding would require a trans-interaction rather than a cis-interac-

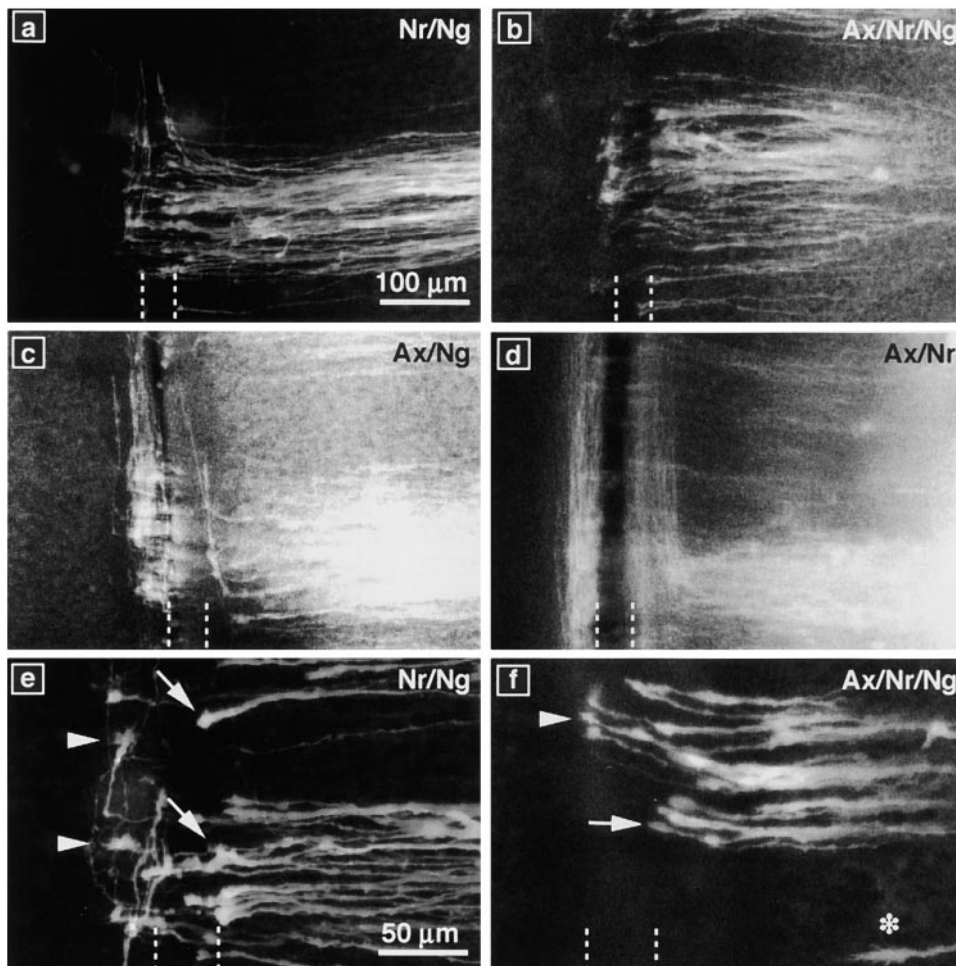


Figure 6. Axonin-1 is important for guidance, but not for elongation of commissural axons *in vivo*. NrCAM and NgCAM provide redundant outgrowth-promoting signals for commissural axons. Axonin-1 mediates axon guidance across the floor plate. In embryos treated with a combination of anti-NrCAM and anti-NgCAM antibodies (a and e), only a few axons managed to elongate along the longitudinal axis. Most axons were stuck at the floor-plate border. Whereas the majority made it across the floor plate, some failed to because of the perturbation of NrCAM interactions. The failure to extend along the longitudinal axis was also found in embryos that were treated with a cocktail of anti-axonin-1, anti-NrCAM, and anti-NgCAM antibodies (b and f). However, consistent with the role of axonin-1 in commissural axon guidance (Stoeckli and Landmesser, 1995), the perturbation of axonin-1 interactions resulted in a significant proportion of commissural axons that were unable to cross the floor plate and, therefore, were found to pile up at the ipsilateral border. Axon elongation along the longitudinal axis was not decreased when either NrCAM or NgCAM could provide a growth-

promoting stimulus. Embryos injected with a combination of anti-axonin-1 and anti-NgCAM (c) or anti-axonin-1 and anti-NrCAM (d) showed no reduction in axon elongation compared with control embryos (not shown). Because both anti-axonin-1 and anti-NrCAM antibodies induced pathfinding errors (Stoeckli and Landmesser, 1995), more fibers were found to turn ipsilaterally in embryos treated with anti-axonin-1 and anti-NrCAM (d) compared with embryos treated with anti-axonin-1 and anti-NgCAM (c). In line with time-lapse observations *in vitro* (Stoeckli et al., 1997), commissural growth cones had a collapsed morphology at the floor-plate border (f, arrow). In contrast, growth cones had a complex morphology at the ipsilateral floor-plate border in the absence of anti-axonin-1 (e, arrows). A difference in growth cone morphology was still observed at the contralateral border (e and f, arrowheads). Note that the change in growth cone morphology is induced upon floor-plate contact only and not simply because of the presence of anti-axonin-1 antibodies, as the growth cone marked with an asterisk in f does not have a collapsed, clublike morphology but exhibits several filopodia. The floor plate is marked with dashed lines in all panels. The longitudinal axis runs vertically. Rostral is to the top, and caudal is to the bottom of each panel. Bars: (a–d) 100 μ m; (e–f) 50 μ m.

tion. To confirm that the topology of the axonin-1/NrCAM binding is different from the axonin-1/NgCAM binding, we stained NrCAM- and axonin-1-expressing myeloma cells with different fluorescent dyes and subjected them to cell aggregation assays (Rader et al., 1993). Mixed aggregates composed of approximately equal numbers of NrCAM- and axonin-1-expressing myeloma cells were formed, indicating a heterophilic trans-interaction of axonin-1 and NrCAM (Fig. 7, a and f). The specificity of cell aggregation was tested in antibody perturbation experiments. In the presence of either anti-axonin-1 Fab or anti-NrCAM Fab, the percentage of cells in aggregates was significantly reduced. In particular, the formation of mixed aggregates was strongly decreased (not shown). In the presence of anti-axonin-1 Fab, the remaining aggregates were mainly

homophilic NrCAM aggregates. Likewise, in the presence of anti-NrCAM Fab, mainly homophilic axonin-1 aggregates were found. In both cases, 10–14% of the cells in the homophilic aggregates were of the other cell type, probably because of unspecific trapping of cells in aggregates. This corresponds to the percentage of untransfected (i.e., wild-type) cells found incorporated in aggregates, when these cells were mixed with NrCAM- or axonin-1-expressing cells, respectively (Fig. 7 f). The formation of homogeneous aggregates composed of NrCAM- (Fig. 7 b), axonin-1- (Fig. 7 c), or NgCAM-expressing myeloma cells (Fig. 7 d) is consistent with previous reports on the homophilic binding capacities of these molecules (Grumet and Edelman, 1988; Mauro et al., 1992; Rader et al., 1993; Buchstaller et al., 1996). Therefore, these results demonstrate

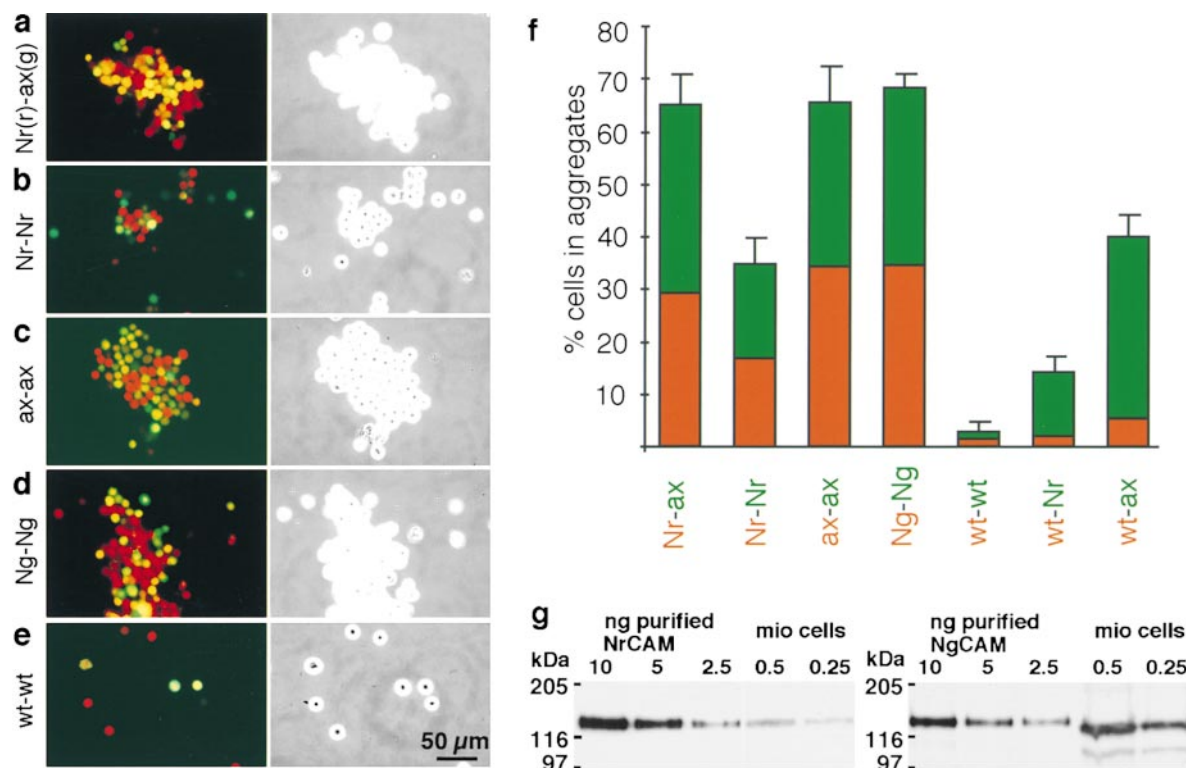


Figure 7. NrCAM and axonin-1 undergo a trans-interaction. A heterophilic trans-interaction between axonin-1 and NrCAM was demonstrated in a cell aggregation assay. Myeloma cells were stably transfected with either NrCAM, NgCAM, or axonin-1. Cells were labeled with different intracellular dyes and incubated as described in Materials and Methods. NrCAM-expressing (red, labeled Nr(r)) and axonin-1-expressing myeloma cells (green, labeled ax (g)) formed large mixed aggregates, demonstrating a heterophilic trans-interaction between axonin-1 and NrCAM (a). Homophilic trans-interactions were observed for NrCAM (b), axonin-1 (c), and NgCAM (d). Wild-type cells (wt), i.e., nontransfected myeloma cells, did not form aggregates (e). A quantitative analysis of the aggregation assays is given in f. The length of the bars represents the percentage of cells found in aggregates (\pm SD). The subdivision of the bars reflects the ratio of the two cell populations. The red part of the bar represents the first molecule (labeled in red) and the green part represents the second molecule (labeled in green). The small contribution of wild-type cells (10–14%) in the wt-Nr and wt-ax combinations indicates an unspecific incorporation of wild-type cells in either homophilic NrCAM or axonin-1 aggregates rather than a specific formation of aggregates. Expression levels of NrCAM and NgCAM were estimated on Western blots (g). A dilution series (10, 5, and 2.5 ng) of purified NrCAM or NgCAM, respectively, was compared with the amount of NrCAM or NgCAM expressed in 0.5 and 0.25 $\times 10^6$ myeloma cells. The bands on the blot were quantified densitometrically for three independent experiments. Representative examples are shown. The expression level of NrCAM was found to be sixfold lower than the level of NgCAM.

unequivocally that axonin-1 and NrCAM from different cells bind each other and mediate cell–cell binding.

The strong heterophilic trans-interaction between NrCAM and axonin-1 found in the present study is in contrast to the absence of a trans-binding between NgCAM and axonin-1 using the same system (Buchstaller et al., 1996). Quantification of NrCAM and NgCAM expression in the myeloma cells by Western blot analysis (Fig. 7 g) revealed a sixfold higher expression level of NgCAM compared with NrCAM. Thus, the absence of an axonin-1/NgCAM trans-interaction cannot be explained by a lower expression of NgCAM compared with NrCAM, but reflects a difference of the two proteins in the topology of their binding to axonin-1.

The Binding Site of Axonin-1 Is Localized on the Four NH_2 -Terminal Ig Domains of NrCAM

For a more detailed characterization of the axonin-1/NrCAM trans-interaction, the NrCAM domains involved in axo-

nin-1 binding and the axonin-1 domains involved in NrCAM binding were identified. For this purpose, domain deletion mutants of both molecules were generated and expressed in COS7 cells that were used for binding studies with axonin-1- or NrCAM-conjugated fluorescent Covaspheres. Cells expressing a heterologous protein on their surface were visualized by indirect immunofluorescence staining.

To identify the domains of NrCAM that are involved in the binding of axonin-1, several domain deletion mutants of NrCAM were generated and expressed in COS7 cells (Fig. 8 c). In the first step, an NrCAM mutant lacking all Ig domains (Δ Ig1-6 NrCAM) and one lacking all FnIII domains (Δ Fn1-5 NrCAM) were constructed. Binding assays with axonin-1-conjugated Covaspheres revealed equal binding to cells expressing Δ Fn1-5 NrCAM and wild-type NrCAM. However, no interaction was found with Δ Ig1-6 NrCAM-expressing cells (Fig. 8 a). Thus, the axonin-1 binding site is located within the Ig domains of NrCAM. For further analysis of the binding domains, we generated NrCAM mutants lacking two Ig domains based on the ob-

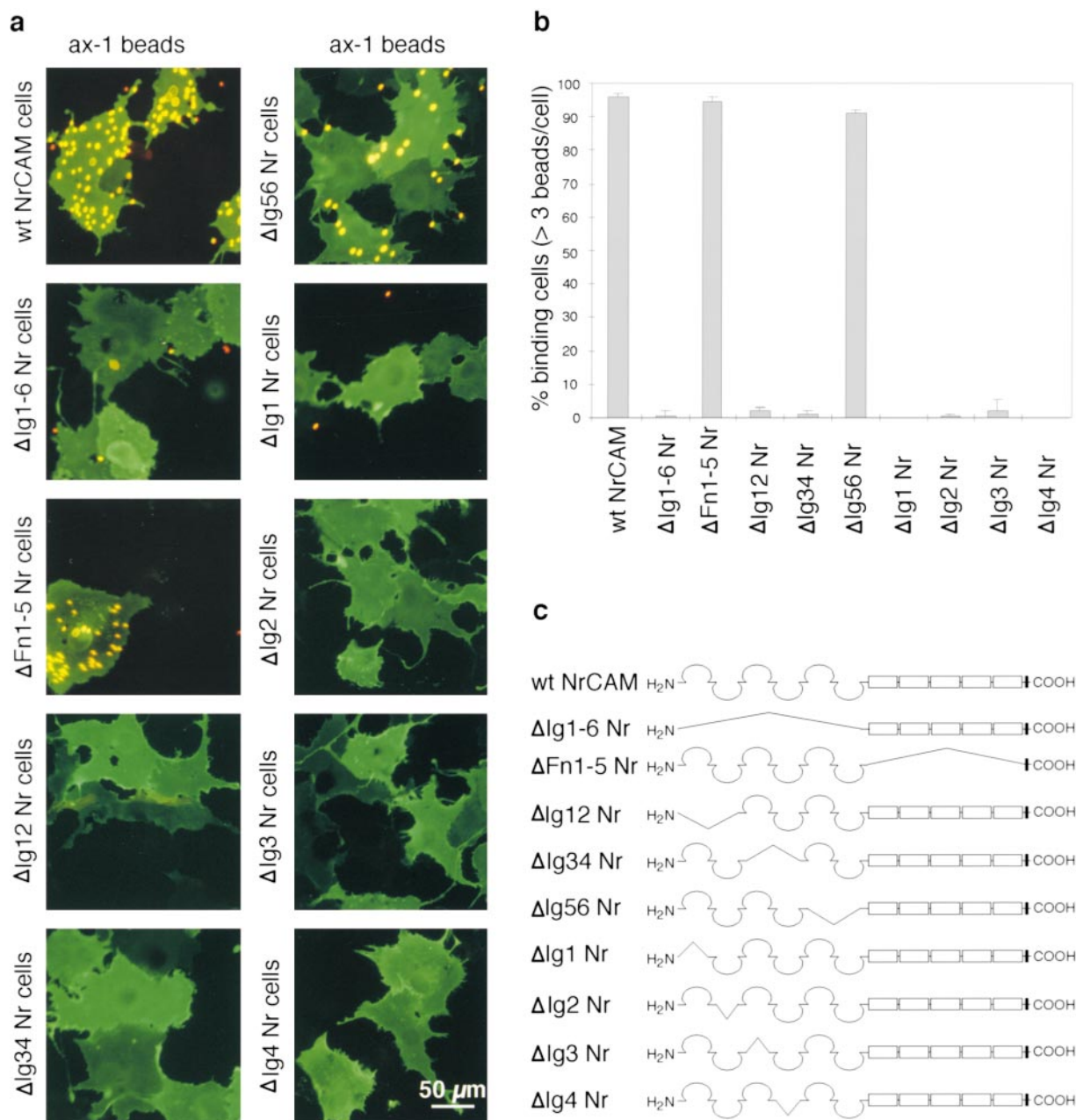


Figure 8. Localization of the axonin-1-binding site on NrCAM. Wild-type and domain deletion mutants of NrCAM were transiently expressed in COS7 cells and tested for their binding capabilities of axonin-1-conjugated Covaspheres (ax-1 beads). NrCAM-expressing cells were identified by indirect immunofluorescence staining using a polyclonal anti-NrCAM antibody and an FITC-labeled secondary antibody. (a) Representative examples of each experimental condition are shown using an FITC-TRITC fluorescence filter. Axonin-1 Covaspheres (TRITC) that bound to NrCAM-expressing cells (FITC) appear in yellow. (b) For each mutant, 100 intensely green-fluorescing cells were analyzed per experiment. Green-fluorescing cells that bound four or more axonin-1 Covaspheres were scored as positive binding cells. Cells that did not express wild-type or mutant NrCAM were used as internal negative controls. Each column corresponds to the mean percentage of binding cells (+SD) of three independent experiments. Wild-type NrCAM expressed in COS7 cells strongly bound axonin-1 Covaspheres. When all Ig domains were deleted (Δ Ig1-6 Nr), no axonin-1 binding was observed, whereas full binding persisted when all FnIII type domains (Δ Fn1-5 Nr) were deleted. From the three double domain mutants within the Ig part (Δ Ig12 Nr, Δ Ig34 Nr, and Δ Ig56 Nr), only Δ Ig56 Nr was able to bind axonin-1 Covaspheres. As none of the single domain deletion mutants Δ Ig1 Nr, Δ Ig2 Nr, Δ Ig3 Nr, and Δ Ig4 Nr bound axonin-1, these findings indicate that the first four Ig domains of NrCAM are all necessary for axonin-1 binding. (c) Schematic representation of wild-type NrCAM and the described domain deletion mutants. NH₂ termini (NH₂) are on the left, and COOH termini (COOH) are on the right. Ig domains are represented by half circles and FnIII type domains by rectangular boxes. The lines indicate the domain deletions.

servation, in many IgFnIII class molecules, that some of the folding units comprise two adjacent domains (Huber et al., 1994; Vaughn and Bjorkman, 1996). We found that Δ Ig12- and Δ Ig34 NrCAM-expressing cells did not bind axonin-1 Covaspheres, whereas full axonin-1 binding was found for Δ Ig56 NrCAM (Fig. 8, a and b). Consequently, we deleted single domains within the segment of the first four Ig domains, either Ig1, Ig2, Ig3, or Ig4, and found that none of these mutants was able to interact with axonin-1-conjugated Covaspheres (Fig. 8, a and b). In summary, the results indicate that all four NH₂-terminal Ig domains of NrCAM are involved in the binding of axonin-1.

The Four NH₂-Terminal Ig Domains of Axonin-1 Are Necessary and Sufficient for NrCAM Binding

We further characterized the axonin-1/NrCAM interaction by localizing the NrCAM binding site on axonin-1. For this, we used the two axonin-1 domain deletion mutants Ig1234 and Δ Ig1234 described previously (Rader et al., 1996). In Ig1234, the four NH₂-terminal Ig domains were coupled directly to the GPI anchor, and Δ Ig1234 was a truncated protein in which the four NH₂-terminal domains were deleted. Ig1234-expressing cells exhibited strong NrCAM binding, comparable to that of cells expressing wild-type axonin-1. In contrast, no NrCAM binding was found with Δ Ig1234 (Fig. 10 a). Thus, we concluded that the location of the binding site for NrCAM was on the segment composed of the first four Ig domains. Based on extensive domain deletion studies of axonin-1 and the recently concluded resolution of the spatial structure of the Ig1-4 segment of axonin-1 (Freigang et al., 2000), the four NH₂-terminal Ig domains of axonin-1 form a structural entity that can maintain its structural and functional integrity only in the presence of all four domains (Rader et al., 1996). Therefore, attempts towards a finer localization of the NrCAM binding site were not made.

The NrCAM and NgCAM Binding Sites on Axonin-1 Are Overlapping but Not Identical

The four NH₂-terminal Ig domains of axonin-1 previously have been identified as the binding site for NgCAM (Rader et al., 1996). To determine the location of the binding sites of NrCAM and NgCAM relative to each other, we carried out perturbation experiments with a selection of mAbs directed to the four NH₂-terminal Ig domains of axonin-1 (Rader et al., 1996). Transiently transfected COS7 cells expressing wild-type axonin-1 were incubated with the different mAbs before the addition of the NrCAM-coated Covaspheres. The same mAbs were subsequently tested for their ability to block NgCAM binding to axonin-1. An axonin-1 mutant lacking the fifth Ig domain (Δ Ig5 axonin-1) was used for NgCAM binding studies, since it had been shown that binding of NgCAM-Covaspheres to membrane-bound axonin-1 strongly increased upon deletion of either the fifth or sixth Ig domain of axonin-1 (Fig. 10 a; Rader et al., 1996). In contrast, no difference of NrCAM binding to wild-type compared with Δ Ig5 axonin-1 was observed (Fig. 10 a). mAb Δ Ig1 specifically blocked NrCAM, but not NgCAM binding to axonin-1, whereas mAb NIg1 blocked binding of both NrCAM and NgCAM

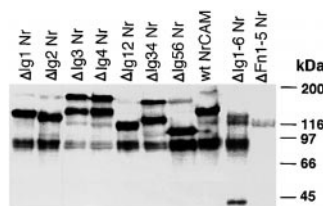


Figure 9. Analysis of the molecular masses of wild-type NrCAM and NrCAM domain deletion mutants. To check the molecular masses and to assess the expression levels of the different mutant NrCAM proteins, COS7 cells were transfected by electropora-

tion and cultivated for 48 h. The solubilized proteins were analyzed by immunoblotting using a polyclonal anti-NrCAM antibody. The bands for wild-type NrCAM were as expected: a weak band of ~200 kDa, corresponding to the small proportion of uncleaved protein; an intense double band of 125/135 kDa, representing the fragment comprising all Ig domains and two and a half FnIII domains; and a third intense band of 80 kDa, corresponding to the COOH-terminal rest of the protein. This 80-kDa fragment and two additional bands of variable molecular mass (corresponding to the uncleaved protein and the cleaved extracellular fragment) were observed in all domain deletion mutants except for Δ Fn1-5 NrCAM. This mutation lacks all FnIII domains and, thus, also lacks the cleavage site. The molecular masses of the uncleaved proteins of the different single and double domain deletion mutants were ~175 and 160 kDa, respectively. No differences among the single domain mutants and the double domain mutants were detectable. However, more variation was found among the molecular masses of the cleaved extracellular fragments. It is possible that these differences were due to a variability of the better resolution of the gel for lower molecular masses. Interestingly, we found that the proportion of uncleaved and cleaved protein varied strongly between the different single and double domain deletion mutants. All mutants lacking either the third or the fourth Ig domain (Δ Ig3 NrCAM, Δ Ig4 NrCAM, and Δ Ig34 NrCAM) displayed an unusually high portion of uncleaved protein and relatively high molecular masses for the large extracellular fragment.

(Fig. 10 b). The mAb X9H8, which blocks NgCAM binding (Rader et al., 1996), did not interfere with NrCAM binding (Fig. 10 b). These results indicate that the NrCAM and NgCAM binding sites on axonin-1 are distinct, but are either close together or overlapping.

Discussion

We have shown that the interaction of growth cone axonin-1 with NrCAM in an appropriate substratum combination in vitro (Fig. 11 b) or expressed by floor-plate cells in vivo (Fig. 11 d) elicits a guidance response of commissural axons without affecting axon extension (Fig. 11, a and c). The instructive signal allows the growth cone to discriminate between two alternative pathways with equal growth-promoting capacities.

The Choice between Two Equally Potent Growth-promoting Substrata Involves a Discriminatory Mechanism Activating an Instructive Signal

Commissural axons on alternating stripes of NrCAM/NgCAM and NgCAM substratum exhibited a clear preference for the mixed substratum. The preferred growth on NrCAM/NgCAM stripes was not due to a higher growth-promoting activity, as measurements of neurite length re-

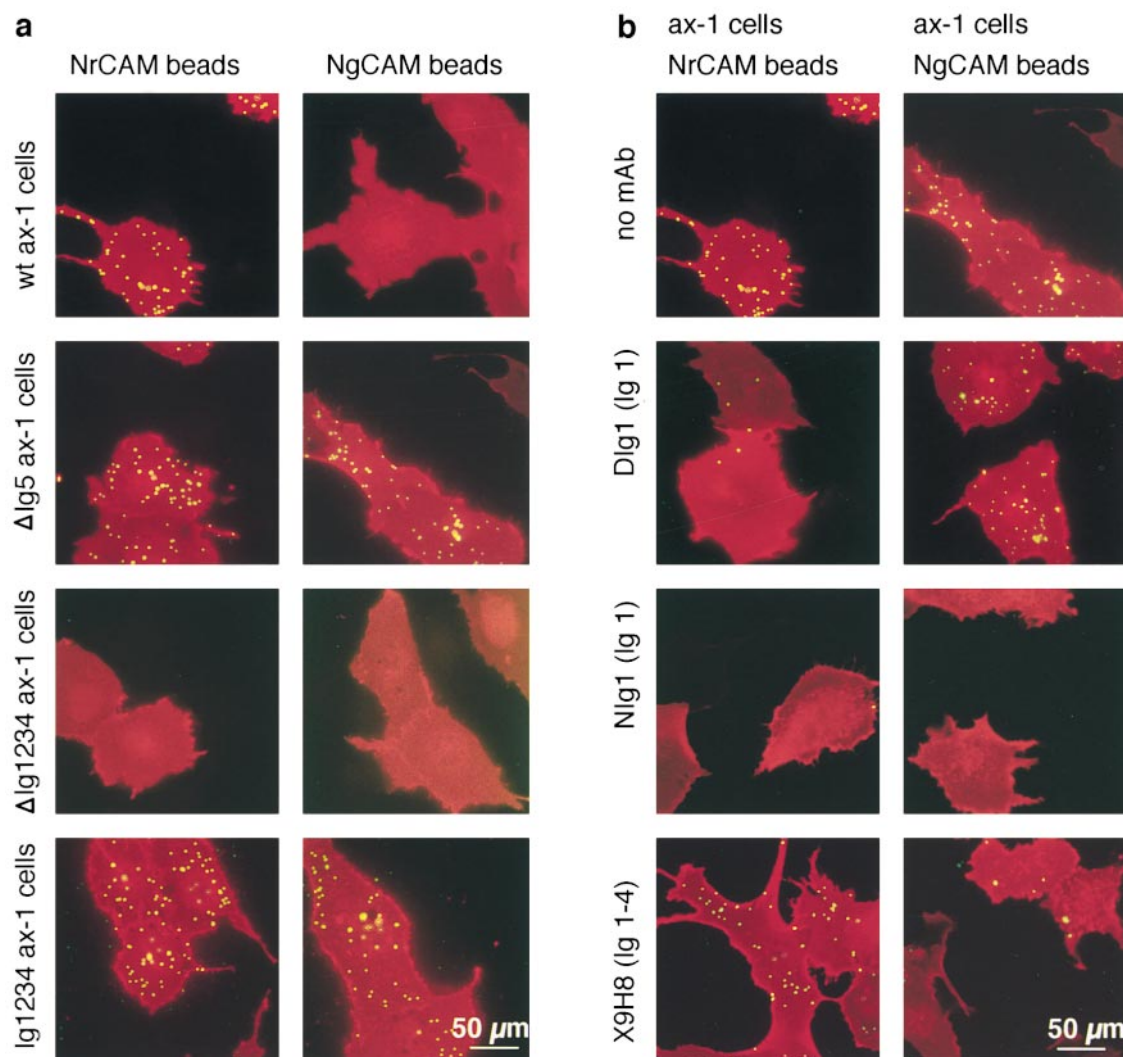


Figure 10. Localization of the NrCAM-binding site on axonin-1. To localize the binding site of NrCAM on axonin-1, mutant and wild-type axonin-1 were transiently expressed in COS7 cells. Covaspheres coated with NrCAM (left) or NgCAM (right) bound to COS7 cells are shown in a. (first row) Only NrCAM but not NgCAM Covaspheres bind to the COS7 cells expressing wild-type axonin-1 (ax-1 cells). Both types of Covaspheres were found to bind to the COS7 cells expressing an axonin-1 mutant lacking the fifth Ig domain (Δ Ig5 ax-1 cells, second row), but not to cells expressing a mutant lacking the Ig domains 1–4 (Δ Ig1234, third row). The first four Ig domains (Ig1234) were sufficient to bind both types of Covaspheres (fourth row). COS7 cells expressing wild-type or mutant axonin-1 were visualized by indirect immunofluorescence using a polyclonal rabbit anti-axonin-1 serum and Texas red-conjugated donkey anti-rabbit IgG. The binding site of NgCAM on axonin-1 was mapped to the conglomerate formed by the first four Ig domains of axonin-1 by Rader et al. (1996). The same axonin-1 mutants used here showed that the binding site for NrCAM is also localized on the first four Ig domains. (b) Because NgCAM-Covaspheres did not bind to COS7 cells expressing wild-type axonin-1, we used COS7 cells expressing the Δ Ig5 variant of axonin-1 to compare NrCAM- and NgCAM-Covaspheres binding after incubation of the cells with the different mAbs. Antibody DIg1, which recognizes an epitope on the first Ig domain of axonin-1, blocked binding of NrCAM- but not NgCAM-Covaspheres (second row). The antibody NIg1, which recognizes a different epitope on the first Ig domain of axonin-1, blocked binding of both types of Covaspheres (third row), whereas X9H8, which recognizes an epitope formed by the Ig domains 1–4, blocked only the binding of NgCAM- but not NrCAM-Covaspheres (fourth row). In perturbation experiments with the mAbs against axonin-1, COS7 cells expressing wild-type or mutant axonin-1 were visualized by indirect immunofluorescence using Cy3-conjugated donkey anti-mouse IgG.

vealed that the combination of NrCAM and NgCAM did not enhance neurite outgrowth compared with NrCAM or NgCAM alone. Complementary to this observation, we found that the preference-generating process can be blocked without a decrease in neurite length. The same anti-axonin-1 antibodies that abolished preference for the mixed NrCAM/NgCAM substratum did not interfere with neurite growth. Thus, we concluded that the preference-

generating effect of the axonin-1/NrCAM interaction is not mediated via enhancing neurite outgrowth on the mixed NrCAM/NgCAM substratum, but rather represents an instructive guidance signal, by which the growth cones' response is changed in favor of one substratum and against the other.

The conclusions drawn from the in vitro assays were tested in a series of in vivo perturbation assays of commis-

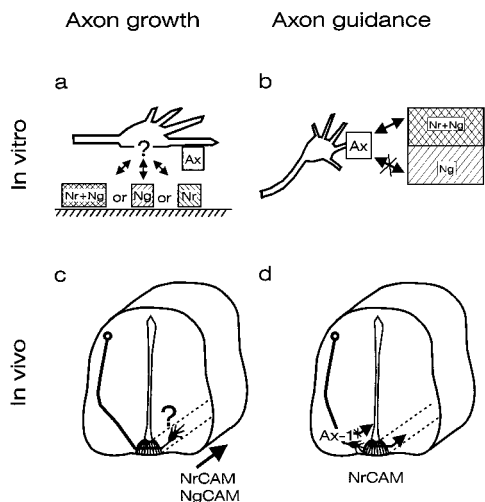


Figure 11. An axonin-1/NrCAM interaction is crucial for guidance of commissural axons, but is not involved in their growth promotion. Based on our *in vitro* and *in vivo* studies, we conclude that an axonin-1/NrCAM interaction is crucial for guidance but not for the growth of commissural axons. Axon guidance is not achieved via the selection of the most growth-promoting substratum, but it depends on an instructive signal to the growth cone that is mediated by axonin-1. a and c summarize our results showing that neurite extension is independent of axonin-1 both *in vitro* (a) as well as *in vivo* (c). In both situations, axon extension is mediated by the interaction of an unknown growth cone receptor with NrCAM and NgCAM. The growth-promoting signals derived from NrCAM and NgCAM appear to be redundant, as neurite lengths were the same on an NrCAM, an NgCAM, and a mixed NrCAM/NgCAM substratum in our stripe assay (b) without decreasing neurite length. Similarly, after injection of anti-axonin-1 antibodies, commissural axons failed to cross the floor plate without exhibiting any decrease in their growth capacity. Although the molecular interactions *in vivo* are far more complex than the situation mimicked in our *in vitro* assay, the choice of commissural axons to grow on the mixed NrCAM/NgCAM substratum (b) and to grow across the midline by entering the floor plate *in vivo* (d) have in common that axonin-1 is the key molecule triggering the growth cone's decision.

sural axons. Obviously, a choice in an *in vitro* system cannot be the same as the choice *in vivo*, where a complex environment provides a variety of possible interactions. Furthermore, the floor plate does not express NgCAM. However, the two choice situations bear in common that an interaction between growth cone axonin-1 and substratum NrCAM is an essential element in the observed preference reaction. Therefore, we found it appropriate to test whether the axonin-1/NrCAM interaction had the character of an instructive signal without affecting axon growth also at this well characterized choice point *in vivo*. Indeed, we found that the effect of axonin-1 in commissural axon guidance was independent of axon elongation. Both NrCAM and NgCAM have neurite outgrowth-promoting activities

that are equivalent and redundant. Masking either NrCAM or NgCAM alone did not decrease neurite elongation. However, if both NrCAM- and NgCAM-derived growth signals were abolished by concomitant injection of anti-NrCAM and anti-NgCAM antibodies, neurite growth in the longitudinal axis was strongly inhibited (Fig. 6, a and e). Consistent with the hypothesis that axonin-1 mediates a guidance signal for commissural axons without promoting their growth, the injection of anti-axonin-1 together with either anti-NgCAM (Fig. 6 c) or anti-NrCAM antibodies (Fig. 6 d) did not reduce neurite length. In both cases, neurites extended along the longitudinal axis over the same distance as neurites in control embryos, although a significant proportion of the axons committed pathfinding errors and extended along the ipsilateral rather than the contralateral border of the floor plate. When NgCAM is masked (Stoeckli and Landmesser, 1995) or the mammalian homologue L1 is inactivated (Cohen et al., 1998), no effect on commissural axon guidance in the spinal cord was found, which is consistent with the idea that NrCAM would still be able to provide a signal for growth and for guidance.

Based on these results, we concluded that the floor plate and the longitudinal tract display their full growth-promoting activities for commissural axons also in the absence of axonin-1/NrCAM interactions. Therefore, the guidance instruction given to the commissural growth cones by the axonin-1/NrCAM interaction *in vivo* is mediated by a mechanism distinct from axon growth promotion. This result is interesting in light of recent findings published by Lustig et al. (1999), demonstrating that axonin-1 is the axonal receptor mediating neurite outgrowth from peripheral ganglia on an NrCAM substratum. Obviously, the contributions of individual CAMs to neurite growth and guidance are context dependent. Whereas an individual CAM can be involved in growth promotion in one neuronal population, it is mediating a guidance signal without influencing neurite extension in another situation. Thus, whereas axonin-1 is providing an instructive guidance signal for commissural axons, without affecting axon extension, NrCAM is involved in both axon guidance (via interaction with growth cone axonin-1) and axon growth (via interaction with an unknown growth cone receptor; Fig. 11). NgCAM is involved in neurite extension only without affecting guidance. Because L1, the mouse homologue of chicken NgCAM, was shown to have an effect on pathfinding of corticospinal axons (Cohen et al., 1998), the role of CAMs has to be characterized in detail for every neuronal population.

In summary, the results of the present study demonstrate that *in vivo* and *in vitro* signals for guidance are distinct from signals for growth. However, the distinction between growth and guidance is not a characteristic function associated with a particular CAM, but rather reflects the role of a CAM in the context of a specific neuronal population.

The Enlargement of the Growth Cones in Our In Vitro Assay Correlates with In Vivo Observations of Growth Cone Size at Choice Points

The growth cones expressing a preference in our *in vitro*

choice assays were approximately twice as large as the ones not expressing a preference (294 versus 145 μm^2). The growth cone size was not determined by the substratum alone. Among the growth cones on mixed NrCAM/NgCAM, the enlarged phenotype was assumed only by those that had chosen NrCAM/NgCAM versus alternating NgCAM and, thus, showed the preference reaction. Growth cones on homogeneously coated NrCAM/NgCAM or on NrCAM/NgCAM stripes alternated with NrCAM were not enlarged. The growth cone enlargement was also not found on alternating stripes of NrCAM/NgCAM and NgCAM, when the preference was abolished by the presence of anti-axonin-1 antibodies. The strict coincidence of growth cone enlargement and preference reaction suggests that the two phenomena are functionally connected. Concurrent conclusions were drawn from observations *in vivo*, where morphological changes of growth cones reacting to particular choice points along their path have been described in fixed and living tissue (Godement et al., 1994; Mason and Wang, 1997). Whereas growth cones have a simple morphology while they are advancing along fiber tracts, they assume a more complex shape at choice points, such as the optic chiasm (Godement et al., 1994; Mason and Wang, 1997) and the floor plate (Bovolenta and Dodd, 1990). In line with our observations *in vitro*, where growth cone enlargement was strictly correlated with a decision for one substratum and against the other, growth cone size was shown to be much larger for growth cones in the floor plates, the decision region, compared with the ipsilateral neuroepithelium (Bovolenta and Dodd, 1990).

Binding of Axonin-1 to NrCAM Can only Be Established when Axonin-1 Is Not Bound to NgCAM

The substratum combination that was found to elicit a preference/enlargement response in commissural axons *in vitro* is distinct from the not yet known substratum combination determining their pathway choice *in vivo*. However, the two reactions have a common denominator. In both situations, a trans-interaction of growth cone axonin-1 and substratum NrCAM is an essential element in the initiation of the preference response. Moreover, in both situations, the interruption of the axonin-1/NrCAM interaction abolishes the preference and results in an indiscriminate growth on the available substrata.

The preference-generating interaction between growth cone axonin-1 and substratum NrCAM is one of several possible interactions for both axonin-1 and NrCAM. Axonin-1 has been shown to undergo a homophilic trans-interaction (Rader et al., 1993) and to form a cis-interaction with NgCAM (Buchstaller et al., 1996). NrCAM interacts homophilically (Mauro et al., 1992) and heterophilically with F11 *in trans* (Morales et al., 1993; Volkmer et al., 1996). In addition, several other ligands have been identified for both, with unknown cellular topology (for reviews see Grumet, 1997; Sonderegger, 1997). With myeloma cells expressing axonin-1 or NrCAM we have demonstrated that axonin-1 and NrCAM establish a strong trans-interaction across the intercellular space. This is in contrast to the interaction between axonin-1 and NgCAM, which is only established between molecules located in the same membrane (Buchstaller et al., 1996). Using the same domain

deletion approach as previously for NgCAM (Kunz et al., 1998), the axonin-1-binding site of NrCAM was localized on the first four Ig domains. Studies with truncated forms of axonin-1 revealed that the binding site for NrCAM is located on the domain conglomerate formed by the first four Ig domains. The same location was previously found for the binding site of NgCAM (Rader et al., 1996). In the same study, evidence suggested that monomeric wild-type axonin-1 is bent back at a hinge located in the middle of the molecule giving it a horseshoelike structure. Because of this structure, the conglomerate of NgCAM-binding domains is located close to the membrane and binding of NgCAM across the extracellular space would not be possible. An axonin-1 mutant that lacks the fifth Ig domain and, therefore, is thought to have an extended structure, binds NgCAM presented on the surface of Covaspheres (Rader et al., 1996). In contrast, we found that strong binding of NrCAM was observed with both cells expressing wild-type and mutant axonin-1 lacking the fifth Ig domain. We concluded that NrCAM can bind axonin-1 across the extracellular space regardless whether it is presented in a horseshoelike or in an extended conformation. Thus, the topological requirements for axonin-1 for the axonin-1/NrCAM interaction are clearly distinct from those needed for the axonin-1/NgCAM interaction. The relative location of the binding sites for NrCAM and NgCAM on the conglomerate of domains Ig1-4 of axonin-1 was addressed by a perturbation study with mAbs. Based on the results, we concluded that the binding areas for NrCAM and NgCAM on the conglomerate of domains Ig1-4 of axonin-1 are distinct, but overlapping, and may result in a mutual exclusion for axonin-1/NrCAM and axonin-1/NgCAM binding.

The establishment of an axonin-1/NrCAM interaction at the expense of an axonin-1/NgCAM cis-interaction could generate the intracellular signals regulating the directional growth of the growth cone at the floor-plate border. The molecular mechanism by which the axonin-1/NrCAM interaction generates preference in growth cones that are in contact with NgCAM and NrCAM is at present not known. We have previously found that intracellular signaling of axonin-1 changes depending on its interactions. Analyses of kinases associated with axonin-1 and NgCAM revealed that monomeric axonin-1 is associated with the tyrosine kinase fyn, whereas monomeric NgCAM is associated with a casein kinase II (Kunz et al., 1996). In low density cultures without neurite-neurite contacts, the axonin-1-associated fyn activity was high and the NgCAM-associated casein kinase II activity was low. When neurites formed fascicles, axonin-1 and NgCAM were found in increasing quantities as heterodimeric and heterotetrameric complexes. The formation of these complexes at the sites of neuritic membrane contacts in the fascicles was accompanied by a switch in intracellular signaling. The signals associated with axonin-1 and NgCAM changed in the opposite direction. Axonin-1-associated fyn was reduced and NgCAM-associated kinases were increased. Based on observations that tubulin polymerization correlates with tyrosine phosphorylation (Thomas et al., 1995), and that a stabilization of microtubules correlates with phosphorylation of MAP1B by casein kinase II (Ulloa et al., 1993), we speculated that a stabilization of the membrane contacts

between different neurites in fascicles might be achieved via a decrease in fyn activity and an increase in the activity of the NgCAM-associated kinases. It is possible that the binding of growth cone axonin-1 with floor-plate NrCAM reverses the dimerization of axonin-1 with NgCAM, and, thus, its integration into heterotetrameric complexes. This could result in the flexibility of commissural axons to make their turns at both floor-plate entry and exit sites.

Conclusions

Growth cones at choice points decide for a particular pathway and against another based on molecular interactions between growth cone receptors and molecular cues encountered at the choice point. Our observations in vitro and in vivo provide evidence that, for commissural axons, the growth cone receptor mediating correct guidance is axonin-1. In both the in vitro model and the in vivo situation, the interaction between growth cone axonin-1 and substratum NrCAM is not involved in axon elongation, but provides the decision making guidance signal, resulting in the preference for one pathway. Growth cones show a marked enlargement dependent on the preference reaction rather than on the substratum both in vitro and in vivo. Although our in vitro model for a choice point for commissural axons is highly simplified using only two selected molecules, NrCAM and NgCAM, as substrata, it exhibits characteristic features associated with the decision of commissural axons at the floor plate in vivo, and, therefore, may be an excellent model for future studies of the molecular mechanisms that determine the growth cone's decisions at choice points.

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